

## Genetic Mapping of the Duffy Binding Protein (DBP) Ligand Domain of *Plasmodium vivax* from Unstable Malaria Region in the Middle East

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**Abstract.** The region II of *Plasmodium vivax* Duffy binding protein (PvDBP-II) contains the critical binding residues, which is a major target for development of naturally acquired immunity. Several studies showed sequence polymorphisms in PvDBP-II, which may inhibit antibodies recognition. Therefore, in this study the level of PvDBP-II polymorphism within and among *P. vivax* populations from re-emerged areas in north and endemic areas in south of Iran were evaluated by sequencing analysis in 75 isolates for the first time. Fourteen non-synonymous and one synonymous mutations were identified and none of the amino acid substitutions were directly involved in erythrocyte binding. Only 6 out of 14 detected mutations have been found among northern isolates, including D384G, R390H, N417K, L424I, W437R, and I503K. In total, two and nine different variants have been identified among northern and southern isolates, respectively. High association of the amino acid frequencies for codons 417, 437, and 503 were found among northern (85% for trio association and 100% for N417K with W437R), and southern (36% for trio association and 98% for N417K with W437R) samples. Polymorphisms at positions R308S, K371E, D384G, K386N, R390H, N417K, L424I, W437R, and I503K were identified from Iran and diverse geographic areas; however, mutation at position F306L was only reported from Asian malaria endemic areas. It is suggested that to develop polyvalent vaccine against *P. vivax* infection, it is better to incorporate the common and high prevalent allelic variants of the antigen that were reported from different malaria endemic regions.

### INTRODUCTION

*Plasmodium vivax* remains a public health problem in many parts of the tropical world, including South and Southeast Asia, the Middle East, North Africa, the Western Pacific, Central and South America.<sup>1-4</sup> With the emergence of *P. vivax* resistance to current anti-malarial drugs,<sup>5</sup> the development of vaccines are being considered. To achieve this goal, molecules that are involved in blocking erythrocyte invasion play an important role and therefore a better understanding of the molecular interactions between the parasite ligand and the red blood cell (RBC) receptor is required. *Plasmodium vivax* is dependent on binding of the Duffy binding protein (DBP) to the Duffy antigen receptor for chemokines (DARC) on erythrocyte.<sup>6</sup> The parasite ligand is a member of the Duffy binding-like (DBL) erythrocyte binding protein (EBP) family<sup>7</sup> expressed in the micronemes of *P. vivax* merozoites.<sup>7</sup> The functional binding domains of DBL lie in region II, the conserved cysteine-rich region.<sup>8-10</sup> This region occurs in the 330 amino acid region II (DBP-II), which has been shown to contain the critical binding motifs,<sup>8,11</sup> and have been mapped to a 170 amino acid region between cysteins 4 and 7.<sup>12</sup> These cystein residues are conserved, whereas other amino acids are highly polymorphic.<sup>13,14</sup>

*Plasmodium vivax* DBP-II (PvDBP-II) is also a major target for development of naturally acquired immunity.<sup>15</sup> Previous study showed that individuals that lack the Duffy surface antigen on their erythrocytes are completely resistant to invasion by *P. vivax* malaria, making the parasite ligand an important vaccine candidate.<sup>6</sup> In addition, the study by Zimmerman and others, in Papua New Guinea, showed reduced susceptibility to *P. vivax* infection in heterozygous carriers of a Duffy-negative allele compared with wild-type homozygotes.<sup>16</sup>

These observations suggest that invasion of human erythrocytes by *P. vivax* requires the interaction between DARC and PvDBP; therefore, complete or partial disrupting access to the Duffy antigen reduces the ability of the parasite to invade new erythrocytes and may constrain *P. vivax* parasitaemia.<sup>17</sup> However, a recent report has described the transmission of *P. vivax* to a Duffy negative population in Kenya, suggesting that *P. vivax* could have alternative invasion pathways, although it is rare and no other means of invasion have been identified yet.<sup>18</sup>

Regarding genetic variation, extensive sequence polymorphisms in PvDBP-II have been reported in *P. vivax* field isolates,<sup>13,14,19</sup> but the contact residues that form the recognition site within this region appear to be invariant.<sup>20,21</sup> Analysis of sequence polymorphism data suggests that there are two stretches of amino acids within PvDBP-II, which contain clusters of polymorphic residues.<sup>22</sup> These amino acids lie on the surface opposite to the recognition site and naturally acquired antibody responses are directed against regions distal to the proposed recognition site.<sup>23</sup> Therefore, the naturally acquired antibodies against PvDBP-II might be directed against regions of the protein that are distal to the binding site, which is polymorphic.<sup>20,22,23</sup>

The dependence of *P. vivax* on interaction with the Duffy antigen for the crucial step of junction formation during invasion provides a unique opportunity for development of an effective intervention strategy against *P. vivax* infection. Analysis of PvDBP showed that this region contains a highly polymorphic sequence<sup>13,14,24-26</sup> and might interfere with its use in vaccine development. Therefore, understanding the nature and origin of this polymorphism present in this antigen among global *P. vivax* isolates is a key feature for vaccine development. Although the available information was obtained from the Papua New Guinean, Colombian, Korean, and Brazilian isolates,<sup>13,14,25,26</sup> no similar published data exist from the Middle East, where the *P. vivax* isolates are prevalent. Furthermore, possessing this data may resolve the determinants of gene flow and patterns of genetic diversity in malaria endemic regions,

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from east to west and vice versa. Therefore, in the current study, polymorphism of PvDBP-II among Iranian isolates was researched to generate useful information on PvDBP-II from the Middle East, which is required for rational design of vaccine against *P. vivax*. We were also interested in identifying the potential allelic families of *pvdgp* gene in this part of the world.

## MATERIALS AND METHODS

**Study areas and *P. vivax* isolates.** Samples were collected on admission from patients diagnosed with vivax malaria in two different endemic regions.<sup>27</sup> The tropical south-eastern endemic area encompasses three provinces: Sistan and Baluchistan, Hormozgan, and the tropical part of Kerman. Transmission is year-round with two peaks, the first in May to August with *P. vivax* as the predominant species, and the second peak from October to November when both *P. falciparum* and *P. vivax* infections are generally equally recorded. In 2007, ~16,489 malaria cases were reported in Iran, 90% of which were microscopically diagnosed as *P. vivax*, and the remainder as *P. falciparum*. Most (94%) of the cases were recorded in the southeastern provinces. The isolates ( $N = 150$ ) were collected from *P. vivax*-infected patients from Chabahar District in Sistan and Baluchistan, during 2000 to 2007. Demographic information of the patients is shown in Table 1.

The second study region was an area of resurgent malaria, Pars Abad in Ardebil province, in the temperate northern area. Malaria reappeared in this region after 20 years, following a large displacement of people from the Republic of Azerbaijan and to some extent from Armenia in 1994. The transmission occurs only from June to October when the average temperature is sufficiently high to allow parasite development in the anopheline vectors. In 2003, ~550 malaria cases were reported from north and all were microscopically diagnosed as *P. vivax*. The isolates ( $N = 100$ ) were collected from symptomatic *P. vivax*-infected patients who sought treatment at the Malaria Clinic in Pars Abad, during the transmission seasons of 2000 to 2003. Malaria came under control in northern Iran in 2003 through a multidisciplinary strategy and implementation of inter-country (conducting border-meetings and expert visits in Iran and Republic of Azerbaijan) and national training courses on vector control management and microscopic diagnosis of malaria vectors and parasites. Accordingly, the main technical interventions used in this region were based on prompt and effective treatment of malaria cases and prevention of malaria by reducing exposure to infective mosquito bites.

All 250 *P. vivax* clinical isolates were diagnosed by light microscopic examination of Giemsa-stained blood smears. A 1-mL blood sample was then collected on admission after informed consent from adults or the parents or legal guardians of children. This study was approved by the Ethical Review Committee in Pasteur Institute of Iran.

**Parasite DNA extraction and molecular analysis of *Pvdgp-II* gene.** Parasite genomic DNA from blood stage

parasites was obtained by phenol/phenol-chloroform extraction and ethanol precipitation from 200  $\mu$ L of whole blood as described previously.<sup>28</sup> The DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and kept at  $-20^{\circ}\text{C}$  until use. Amplification by polymerase chain reaction (PCR), encompassing the cystein 4 to 7 of *Pvdgp-II* gene corresponding to the base pairs 592–1764 (amino acid [aa] 198–588 of the DBP-II) (Sal-I strain, M61095), has been conducted by the following oligonucleotide primers, which were designed in our laboratory:

dbpF: GATTATGAGACATCTAGCAATGG

dbpR: CGATAAGTCAGCCTGTAGATAGT

The high fidelity Taq DNA polymerase (Invitrogen, Carlsbad, CA) was used in PCR to reduce possible nucleotide misincorporation. The cycling conditions were  $94^{\circ}\text{C}/\text{minute}$  followed by 35 cycles of  $94^{\circ}\text{C}/\text{minute}$ , annealing at  $60^{\circ}\text{C}/\text{minute}$ , and extending at  $72^{\circ}\text{C}/\text{minute}$  with a final primer extension at  $72^{\circ}\text{C}/10$  minutes.

Amplified DNA fragments were analyzed by electrophoresis on agarose gels and visualized on an ultraviolet (UV) transilluminator after ethidium bromide staining.

**Sequencing analysis and phylogenetic analysis.** Sequence analysis was used to identify polymorphisms in the *Pvdgp-II* amplified from 20 and 55 isolates collected from northern and southern Iran, respectively (representing more than 20% of the total examined samples). The PCR products were gel-purified using QIAGEN DNA purification kit (Qiagen, Germany) according to the manufacturer's instructions. Direct sequencing of the DNA fragments was performed in both directions for each PCR product using an ABI 3100 DNA sequencer (Kawsar, Biotech, Iran).

Nucleotide and amino acid sequences were aligned with the corresponding Sal-I (M61095) sequences by using the CLUSTAL X (with manual editing) to identify the common polymorphisms at residues 308, 384, 386, 417, 424, 437, 447, and 503 according to VanBuskirk and others,<sup>29</sup> and alleles were classified based on protein sequences alignments. Nucleotide sequences reported in this article are available in the European Molecular Biology Laboratory (EMBL), GenBank, and DNA Data Bank of Japan (DDJB) databases under accession numbers EU860428–EU860438.

Nucleotide and amino acid sequences were aligned and compared by using CLUSTALX, with the following published sequences: the sequences from 24 Papua New Guinean isolates (PNG) (AF289635–AF289653, AF28980–AF28984, AF291096; AF469530, AF469550)<sup>14,15</sup>; 19 sequences from Colombia (U50575–U50590, DQ156513, U50588, U50589)<sup>13</sup>; 13 sequences from South Korea (AF215737–AF215738, AF220657–AF220664, DQ156522, DQ156515, and DQ156523)<sup>25</sup>; from Brazil<sup>26</sup> (DQ156520), India (DQ156514, DQ156516), Thailand (EF-368159–EF368180, EF379127–EF9135, and EF219451)<sup>30</sup> and

TABLE 1  
Demographic characteristics of the studied patients

	No. of samples	Malaria cases		Mean age, years	Iranian	Nationality			Travel history	
		Male	Female			Afghani	Pakistani	Afghanistan	Pakistan	Inside province
North	150	45%	55%	25	100%	–	–	–	–	3%
South	200	72%	28%	24	60%	10%	30%	3%	17.5%	12%

Indonesia (DQ156521); Bangladesh: (DQ157473); Vietnam: (DQ156518); and Honduras: (DQ156517).

The phylogeny tree was constructed by MEGA version 4.0 program (built with the Neighbor-Joining (NJ) method, pairwise deletion) based on amino acid sequences of PvDBP-II from Iranian isolates.<sup>31</sup> The tree was constructed using region common to all available PvDBP-II amino acid sequences (aa 218–568 of the DBP-II, excluding primer regions and adjacent bases, which did not give reliable sequences).

RESULTS

**Analysis of region II of *P. vivax* DBP.** The *Pvdbp-II* gene was successfully amplified from genomic DNA purified from 250 isolates collected from patients infected with *P. vivax*. The amplified fragments were equal in size and were about 1170 bp for northern and southern isolates. Randomly, 75 isolates (20 from north and 55 from south) were selected for further sequence analysis of *Pvdbp-II* gene.

The amino acid sequence data of PvDBP-II in all 75 sequenced samples demonstrated nucleotide changes, leading to non-synonymous mutations at 14 positions compared with Sal-I sequence (M61095) (Figure 1). 306 [F(tt)/L(ttg)], 308 [R(agg)/S(agt)], 371 [K(aaa)/E(gaa)], 384 [D(gat)/G(ggt)], 385 [E(gaa)/K(aaa)], 386 [K(aag)/N(aat)], 390 [R(cgt)/H(cat)], 398 [S(tct)/T(act)], 404 [T(aca)/R(aga)], 417 [N(aat)/K(aaa)], 419 [I(ata)/M(atg)], 424 [L(tta)/I(ata)], and 437 [W(tgg)/R(cgg)], between cysteines 4 and 7 at positions 291 and 460, respectively, but 503 [I(ata)/K(aaa)]. No mutation was present at position 447 among all Iranian sequenced isolates and all the Cys residues were also conserved. In southern isolates, nucleotide change cgc to cgt made a synonymous mutation in position 378 of PvDBP-II, and one amino acid insertion was observed between amino acids 474 and 475 [L(cta)] in 7 isolates.

Compared with the Sal-I dbp sequence, nine different allelic forms, DBP-I and DBP-VIII, were detected among analyzed samples (Figure 1). The DBP-V and DBP-VI haplotypes were detected among sequenced northern isolates, however all nine haplotypes were found in south. The frequency of haplotype distribution is shown in Figure 2 and the haplotypes DBP-V (85%) and DBP-VI (42%) were the predominant

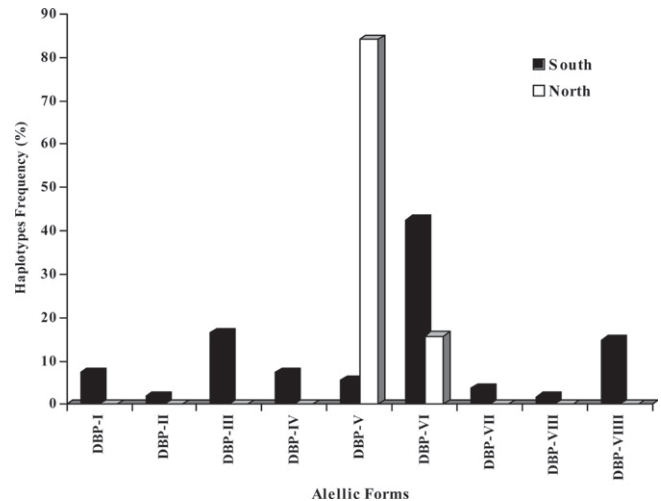


FIGURE 2. Frequency distribution of the different PvDBP-II haplotypes was obtained from 75 *Plasmodium vivax* samples collected from the north and south of Iran. High prevalence of DBP-V (KIRK) and PvDBP-VI (GHK) has been found among northern and southern isolates, respectively.

forms among northern and southern *P. vivax* isolates, respectively (Figure 2).

The frequencies of common variant codons of PvDBP-II compared with the Sal-I DBP sequence isolate among Iranian isolates are shown in Table 2. The variant codons I503K (70.6%), D384G (61.3), and L421I (50.6%) were the most prevalent in Iran.

Analysis of the association of N417K, W437R, and I503K frequencies together revealed that 85% of the northern samples had three mutations, however only 36% of southern isolates showed these trio mutations (data not shown).

**Comparison of PvDBP-II in Iranian isolates with that in other diverse geographic areas.** In this study we found nine variant types in Iranian *P. vivax* clinical isolates, namely after I to VIII. Based on the presence of the commonly variant amino acids 308, 384, 386, 417, 424, 437, 447, and 503 according to VanBuskirk and others,<sup>29</sup> reported in PvDBP-II so far, we found 6 different variant forms (Table 3). The most common variants were D (42%) and B (16.6%) forms, which were reported only from Brazil.<sup>26</sup> The third prevalent allelic

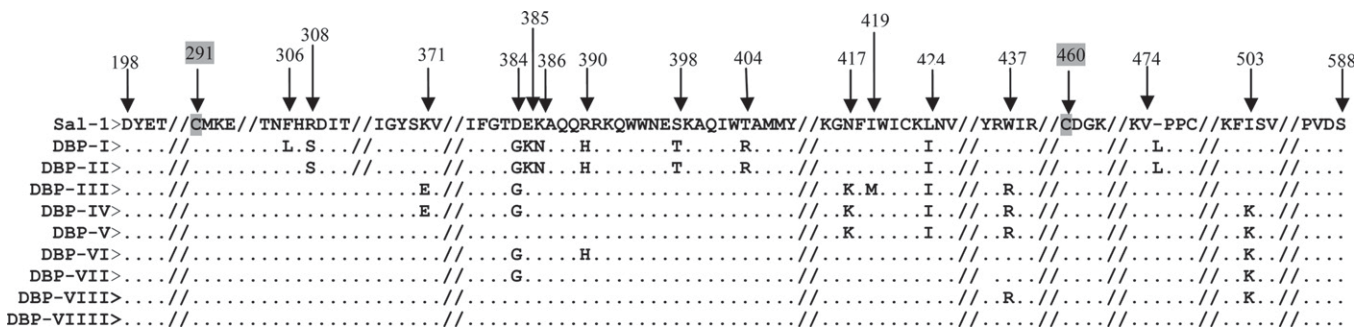


FIGURE 1. Alignment of the nine different PvDBP-II amino acid sequences observed in 75 Iranian isolates. Sequences were compared with Salvador sequences (M61095). The representative PvDBP-II haplotypes are: DBP-I (LSGKNHTRIL, N = 4), DBP-II (SGKNHTRIL, N = 1), DBP-III (EGKMR, N = 9), DBP-IV (EGKRIRK, N = 4), DBP-V (KIRK, N = 20), DBP-VI (GHK, N = 26), DBP-VII (GK, N = 2) and DBP-VIII (RK, N = 1), and DBP-VIII (N = 8) 100% similar to Sal-I sequence type. The // indicate conserved sequences, dots and dashes represent identical residues and deletions, respectively. Cysteines are highlighted. The amino acid residues were numbered according to Fang and others.<sup>36</sup>



TABLE 2  
Distribution of the most commonly variant amino acids in PvDBP-II, among *Plasmodium vivax* isolates from global malaria endemic areas\*

	R308S	D384G	K386N	N417K	L424I	W437R	S447K	I503K
Iran	6.6	61.3	6.6	44	50.6	45.3	0	70.6
Brazil	12.5	85	12.5	27.5	32.5	27.5	0	55
Colombia	0	59	23	47	47	18	0	12
PNG	67	66	8	23	34	26	59	29
South Korea	0	64	5	98	100	100	0	100
India	0	50	0	0	50	0	0	50
Thailand	25.5	74	38.7	38.7	80.6	61.2	0	48.3

\* The variants were compared with Sal-I sequence (M61095). Iranian isolates ( $N = 75$ , present study), Brazilian isolates ( $N = 40$ ),<sup>26</sup> Colombia isolates ( $N = 17$ , U50575–U50590), Papua New Guinean isolates ( $N = 125$ , AF469515–AF469602, AF289635–AF289653, AF289480–AF289483, AF291996, L23069–L23075, U10103–U10107), South Korean isolates ( $N = 42$ , AF215737–AF215738, AF220657–AF220668), Indian isolates ( $N = 2$ , DQ156514, DQ156516), and Thai isolates ( $N = 32$ , EF368159–EF368180, EF379127–EF9135 and EF219451). The residues were numbered according to Fang and others.<sup>26</sup> Residues with  $\geq 50\%$  of prevalence were highlighted in grey.  
PNG = Papua New Guinea.

form was F (14.5%) that was reported from Brazil, PNG, and Colombia. Variant form E (1.8%) seems to be unique to the Iranian isolates (Table 3). The comparison of variant frequency in PvDBP-II between Iranian and Brazilian, Colombian, PNG, South Korean, Thai, and Indian isolates is shown in Table 2. All sequences, but PNG samples, indicated no variability in 477 positions. The I503K (70.6%) and D384G (61.3%) were the two most prevalent variants in our isolates, similar to the results obtained in South Korean isolates (Table 2). Phylogenetic analysis was used to research the associations of PvDBP-II protein with other global sequences. The nine distinct Iranian variant forms were widely distributed among different isolates from distinct geographic regions. The isolates IranS I (DBP-I haplotype) was similar to Indonesia isolate; IranS II (DBP-II) was related to Brazilian and Thai isolates; IranS III (DBP-III) to Thai isolate; IranS IV (DBP-IV) to South Korean isolate; IranS V (DBP-V) to Colombian and Thai isolates; IranS VI (DBP-VI), IranS VII (DBP-VII), and IranS VIII (DBP-VIII) to Colombian isolate; IranS VIII (DBP-VIII) to Indian and Sal-I isolates. The Iranian isolates were distinct from PNG isolates (Figure 3).

## DISCUSSION

The PvDBP is considered to be an important vaccine candidate antigen and several studies showed that the region II of the PvDBP, which contains the critical binding domain, is highly polymorphic.<sup>32</sup> Therefore, the first important step in evaluation of this protein, as a vaccine candidate, is to determine the level of the polymorphism of PvDBP-II within and between populations from different geographic regions.

TABLE 3  
Comparison of the commonly variant amino acids of PvDBP-II in Iranian isolates with that found in other global geographical areas\*

aa haplotypes	308	384	386	417	424	437	447	503	Other areas
A	S	G	N	N	I	W	S	I	Bra, PNG
B	R	G	K	K	I	R	S	I	Bra
C	R	D	K	K	I	R	S	K	Bra, PNG, SK
D	R	G	K	N	L	W	S	K	Bra
E	R	D	K	N	L	R	S	K	–
F	R	D	K	N	L	W	S	I	Sal-I, Bra, PNG, Col,

\* Bra = Brazil; PNG = Papua New Guinea; SK = South Korea; Col = Colombia; Sal-I (M61095); aa = amino acid.

This study was the first report on genetic mapping of Iranian PvDBP-II from the Middle East, where the high prevalence of *P. vivax* infections was reported. The obtained results were in accordance with the previous study that PvDBP-II are diverse within and between populations.<sup>14,26</sup>

Previous study revealed that the polymorphisms at residues reported in the current study were not involved in erythrocyte binding,<sup>13–15,21,26</sup> but they are responsible for parasite escapes from host immune system. The immunity to PvDBP-II is strain-specific in antibody response; therefore, it is important to characterize the allelic forms of the PvDBP-II as a vaccine candidate protein in *P. vivax* parasites originating from global malaria endemic regions, because numerous polymorphisms were reported in these regions. In Iran, as a hypo-meso endemic area, we found nine different haplotypes among 75 sequenced samples, which the majority (eight of them) was reported from diverse geographic areas with different prevalence. However, in highly endemic areas of Thailand and PNG, 25 (for total of 751 samples) and 27 (for total of 30 samples) different PvDBP-II haplotypes were reported, respectively.<sup>30,33</sup> In addition, phylogenetic analysis of PvDBP-II revealed that all nine groups were related to the Brazilian, Colombian, Thai, Indonesian, South Korean, and Indian isolates, but were distinct from PNG. Furthermore, the polymorphisms in variant residues of globally distributed haplotypes were the same but with different prevalence; therefore, this evidence supports that the polyvalent vaccine containing DPB-II may be useful against global *P. vivax* in different malaria endemic regions.

The frequency of N417K, W437R, and I503K variants that are involved in evasion to antibody neutralization was 44%, 45%, and 70.5% in Iranian isolates, respectively. These results were different with frequency of all three variants found in Brazil (N417K, 27.5%; W437R, 27.5%; and I503K, 55%), Colombia (47%, 18%, and 12%, respectively), PNG (23%, 26%, and 29%, respectively), and Thailand (36%, 63% and 56%, respectively).<sup>26,30</sup> The association of the amino acid frequencies for these three codons (417, 437, and 503) previously demonstrated among PNG isolates, was also researched for Iranian isolates. The variant I503K occurred in conjunction with either N417K (49%) or W437R (69%) variants, however, those N417K with W437R occurred in high proportion within Iranian isolates (98.6%). The interesting point is the high prevalence of this association among re-emerged *P. vivax* isolates in north (85% for trio association and 100% for N417K with W437R), which was different from southern isolates (36% for trio association and 98% for N417K with W437R). On the basis of previous study, these three variants are linked polymorphisms, which are responsible for altering the antigenic character of the DBP antigen.<sup>29</sup> Antibody to the variants N417, W437, and I503 (Sal-I) showed higher inhibitory to homologous variant than non-homologues, revealing that polymorphism in these three positions might help the parasite to escape from immune recognition. In other words, they are probably a part of a protective epitope on the DBP ligand that are under immune positive selection. In addition, the prevalence of the polymorphism at residues 417K, 437R, and 503K (85%) were higher in the present study among northern isolates than that of southern (36%).

From the epidemiologic point of view, malaria was reappeared after 20 years in north<sup>27</sup> but it is endemic in south; therefore, most of the northern *P. vivax* samples were isolated from the first exposure patients, but repeated *P. vivax*

infection has a high prevalence among southern samples. Having this fact and a reminder that a single *P. vivax* infection does not induce protective immunity<sup>34</sup> will raise the question of why the prevalence of these mutations, which are a part of a protective epitope on the DBP ligand, is higher among *P. vivax* parasites isolated from the first exposure than those from repeated exposure patients? Two hypotheses can be postulated for this question. First, it could be that these

three variant residues are unlikely to be important in erythrocyte binding alone and polymorphisms in these residues in association with other codons can alter the efficacy of an acquired inhibitory immune response. Second, it might be an indication for a founder effect linked to the introduction of malaria from Azerbaijan and Armenia to Iran and in fact, this hypothesis seems more reliable than the first one. The evidence to support this hypothesis is in the present study 6 out of 14 detected mutations have been found among northern isolates, including D384G, N417K, L424I, W437R, and I503K, and also no insertion has been found among northern samples. This is consistent with the concept of the association of genetic diversity and the levels of transmission as study in highly transmission areas, such as Thailand and PNG, showed 25 and 142 non-synonymous mutations, compared with a low transmission region, such as Iran (14 non-synonymous). In fact the present study was in concordance of our earlier work,<sup>27</sup> in which a level of the genetic diversity in *P. vivax* isolates from northern is lower than those from southern Iran. All of the northern samples were Iranian nationals who had not traveled outside the province within the two months before the survey. However, southern *P. vivax* parasites were isolates from patients with Iranian, Afghani, and Pakistani nationals and many had traveled to and from these three countries. Furthermore, it is possible that these parasites have been disseminated through population movement from south to north, but again no evidence of internal migration between the north and south was noted among our studies samples. The lower genetic diversity in the present study (based on *pvdBP-II*) and our previous work (*pvmSP-I*<sup>27</sup> and *pvcSP*<sup>35</sup>) among northern isolates suggests a founder effect linked to an introduction of malaria from Azerbaijan and Armenia to the northern part of Iran.

However, looking at the total polymorphism in PvDBP-II, we found that polymorphisms at positions R308S, K371E, D384G, K386N, R390H, N417K, L424I, W437R, and I503K were also reported from diverse geographic areas; however, mutation at position F306L was only reported from Asian malaria endemic areas, including Iran (present study),

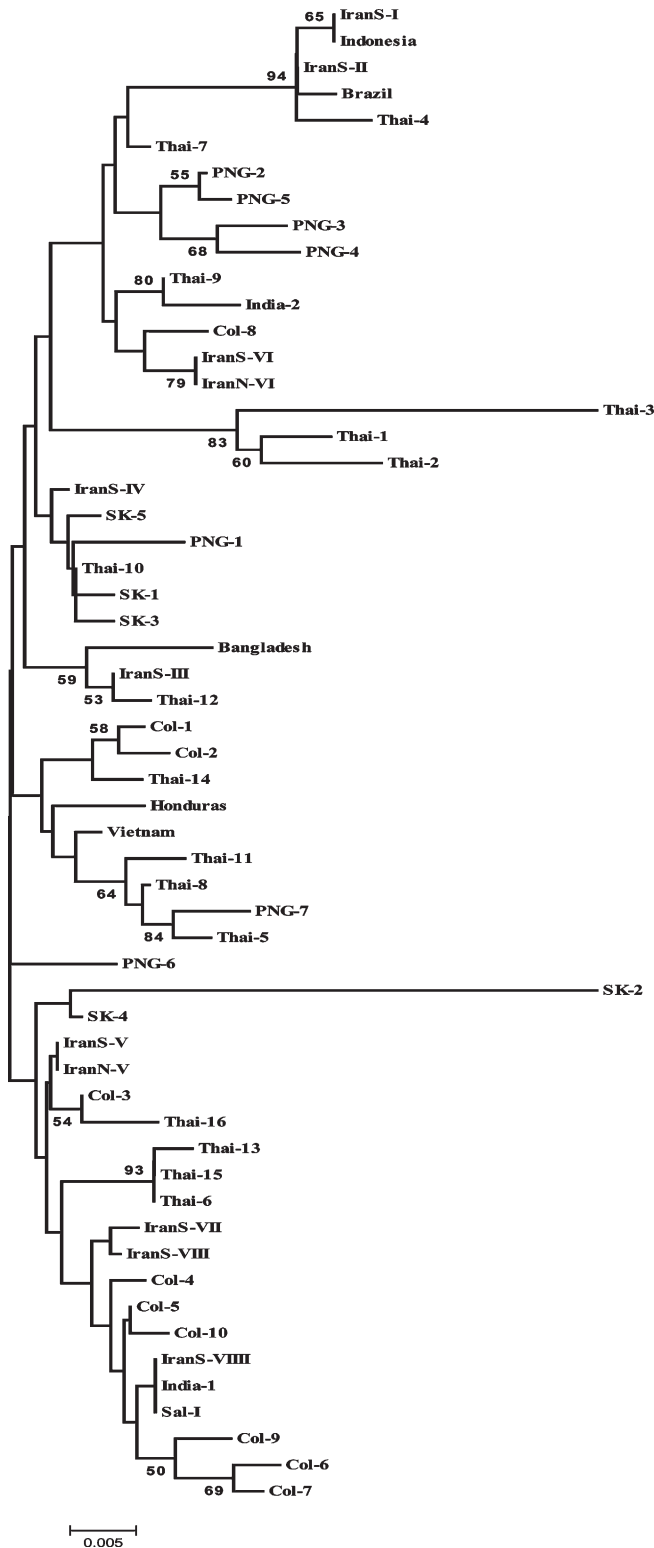


FIGURE 3. Phylogenetic tree was constructed by MEGA 4 program based on the amino acid sequences of PvDBP-II protein from areas with re-emerged (northern) and endemic (southern) malaria Iran and from previously published sequences. The northern samples with different haplotypes are indicated as IranN and southern isolates as IranS with haplotypes numbered I–VIII. The geographic origin of the 45 PvDBP-II published sequences is as follows: Reference strain **Sal-I** (M61095); **Papua New Guinea**: PNG-1 (AF289635), PNG-2 (AF289652), PNG-3 (AF289482), PNG-4 (AF289645), PNG-5 (AF289640), PNG-6 (AF469530), PNG-7 (AF469550); **South Korea**: SK-1 (AF220657), SK-2 (AF220658), SK-3 (AF220662), SK-4 (AF220660), SK-5 (AF220661); **Colombia**: Col-1 (U50577), Col-2 (U50575), Col-3 (U50579), Col-4 (U50576), Col-5 (U50583), Col-6 (U50584), Col-7 (U50585), Col-8 (DQ156513), Col-9 (U50588), Col-10 (U50589); **Thailand**: Thai-1 (EF219451), Thai-2 (EF379127), Thai-3 (EF379128), Thai-4 (EF379129), Thai-5 (EF379130), Thai-6 (EF379131), Thai-7 (EF379134), Thai-8 (EF368165), Thai-9 (EF368171), Thai-10 (EF368172), Thai-11 (EF368174), Thai-12 (EF368175), Thai-13 (EF368176), Thai-14 (EF368178), Thai-15 (EF368179), Thai-16 (EF368180); **India**: India-1: DQ156514, India-2: DQ156516; **Brazil**: (DQ156521); **Bangladesh**: (DQ157473); **Vietnam**: (DQ156518); **Brazil**: (DQ156520); **Honduras**: (DQ156517). The length of the bottom line is proportional to the genetic differences (%). The bootstrap values on the branches are indicated by the number of times in 1,000 replications. Only bootstrap values about 50% are shown.

India (DQ156516), Indonesia (DQ156521), and Thailand (EF368162, EF368169). Positions S393T, T404R, and insertion [L (cta)] between residues 474 and 475 were only shared among Iranian (present study), Indonesian (DQ156521), and Brazilian (DQ1566520) isolates. This comparison suggested that in different areas, the selection pressure might have contributed to these differences. Sharing some variants among Asian and Brazilian isolates indicates that their mutational events might have been apparently independent from their origin.

In summary, genetic research in PvDBP-II among Iranian *P. vivax* isolates showed the genetic diversity as shown in parasite isolates originating from different malaria settings. Therefore, it is suggested that to develop and deploy a polyvalent vaccine against *P. vivax* infection, it is rational to incorporate the common and high prevalent allelic variants of the antigen that were reported from different malaria endemic areas, where *P. vivax* is the most prevalent *Plasmodium* malaria parasite.

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